

AMENDMENTS TO THE SPECIFICATION

Please replace the Sequence Listing with the substitute Sequence Listing submitted herewith.

On page 1, below the title, please insert the following paragraph:

The substitute Sequence Listing for this application is submitted as a text file via EFS-Web, and is incorporated by reference herein. The name of the text file containing the substitute Sequence Listing is "substitutesequencetext.txt". The text file is 16799 bytes (16.4 KB), and was created on October 26, 2009.

Please replace the paragraph at page 2, line 24 to page 3, line 5 with the following paragraph:

The present provides combinatorial protein libraries comprising a plurality of protein species, in which each protein species ~~comprising~~ comprises an A chain of a heteromeric toxic protein into which an insert has been introduced. In accordance with the invention, the insert is a polypeptide of varying amino acid sequence having a length of 2 or more amino acid residues, for example from 3 to 200 amino acid residues; and the insert is introduced into the protease-sensitive loop of the A chain sequence. The ~~result of the~~ introduction of the insert creates an artificial binding domain within the A chain; such that the A chain develops toxic specificity which is independent of and different from the normal specificity associated with the B ~~chains~~ chain binding domains. Screening of the library allows the selection and identification of mutant toxins that are specific for different cell types, including cancer cell types. In one embodiment of the

invention, the combinatorial library comprises protein species that are formed by introducing the insert into a Shiga-like toxin I A chain, for example in the region between amino acids ~~242~~ 248 and ~~261~~ 267, as defined with reference to ~~Seq.~~ SEQ ID No.: 1.

Please replace the paragraphs at page 4, line 28 to page 5, line 4 with the following paragraphs:

Fig. 2A is a schematic representation of the SLT-1 A chain (1-293) with the breast cancer-associated MUC1 epitope PDTRPAP (SEQ ID No.: 8) (control sequence recognized by the mAb Onc M27) inserted between residues 245 and 246 and a 6-Histidine tag (SEQ ID No.: 9) at its N-terminus.

Fig. 2B is a depiction of our SLT-1 A chain-tripeptide library construction where the three key positions of the MUC1 epitope (SEQ ID No.: 32) recognized by the mAb Onc M27 were randomized (XXX region). The tripeptide library was inserted in a naturally occurring loop region of the A chain created by the presence of a disulfide bridge between Cys 242 and Cys 261.

Please replace the paragraph at page 6, line 29 to page 7, line 2 with the following paragraph:

One specific example of the A chain of a heteromeric toxic protein is the A chain of SLT-1 which has the sequence given is ~~Seq.~~ SEQ ID No.: 1. The A chain of SLT-1 comprises ~~of~~ 293 amino acids with the enzymatic(toxic) domain spanning residues 1 to 239 (corresponding to residues 7 to 245 with reference to SEQ ID No.: 1). A protease sensitive loop encompassing residues 242 to 261

(corresponding to residues 248 to 267 with reference to SEQ ID No.: 1) is normally exposed, and is a suitable site for inserting a peptide sequence.

Please replace the paragraph at page 7, line 4 to page 8, line 8 with the following paragraph:

SLT-I is a type II ribosome inactivating protein produced by pathogenic strain of *Escherichia coli* (0157:H7) (24). SLT-I is an AB5 complex of about 70 kD (O'Brien, A. D., and Holmes, R. K., (1987) Shiga and Shiga-like toxins. *Microbiol Rev* 51, 206-220.). The single 32 kD catalytic A subunit is non-covalently associated with a pentamer of five identical 7.7 kD B subunits. The B subunit pentamer recognizes the glycolipid globotriaosylceramide (also known as CD77 or Gb3) on the surface of target cells (Lingwood, C. A. (1993) Verotoxins and their glycolipid receptors. *Adv Lipid Res* 25, 189-211; Jacewicz, et al. (1986) Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J Exp Med* 163,1391-1404). A protease-sensitive loop located between Cys242 and 261 at the C terminus of the A chain is cleaved by furin during cellular routing (Fig. 1). The A chain remains associated with its B subunit pentamer due to an intrachain disulfide bond between Cys242 and Cys261 (corresponding to residues 248 to 267 with reference to SEQ ID No.: 1) as it travels to the ER lumen (Sandvig, et al. (1989) Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from *Shigella dysenteriae* 1. *J Cell Biol* 108, 1331-1343;32. Garred, et al: (1995) Role of processing and intracellular transport for optimal toxicity of Shiga toxin and toxin mutants. *Exp Cell Res* 218, 39-49.). The disulfide bond is finally reduced in the ER lumen and the A1 chain

(first 251 aa) is released and subsequently retrotranslocated to the cytosol where it inactivates ribosomes (O'Brien, et al. (1992) Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr Top Microbial Immunol 180, 65-94.). More specifically, the A chain of SLT-I is a N-glycosidase that catalytically cleaves a specific adenine nucleotide (4324) from the 28S rRNA (Brigotti, et al. (1997) The RNA-N-glycosidase activity of Shiga-like toxin I: kinetic parameters of the native and activated toxin. Toxicon 35,1431-1437). This event leads to the inhibition of protein synthesis by preventing the binding of aminoacyl tRNAs to the ribosome and halting protein elongation. Mutagenesis studies as well as structural analysis performed on the A chains of ST and ricin have delineated key conserved residues involved in catalytic activity (Deresiewicz, et al.(1992) Mutations affecting the activity of the Shiga-like toxin I A-chain. Biochemistry 31, 3272-3280; Ready, et al. (1991) Site-directed mutagenesis of ricin A-chain and implications for the mechanism of action. Proteins 10, 270-278). Residues crucial for catalytic activity of SLT-I are tyrosine 77 (corresponding to residue 83 with reference to SEQ ID No.: 1), glutamic acid 167 (corresponding to residue 173 with reference to SEQ ID No.: 1), arginine 170 (corresponding to residue 176 with reference to SEQ ID No.: 1) and tryptophan 203 (corresponding to residue 209 with reference to SEQ ID No.: 1) (Hovde, et al. (1988) Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I. Proc Natl Acad Sci USA. 85, 2568-2572; Yamasaki, et al. (1991) Importance of arginine at position 170 of the A subunit of Vero toxin 1 produced by enterohemorrhagic Escherichia coli for toxin activity. Microb Pathog 11, 1-9). In addition, binding of the toxin to the cell surface is critical to introduction into the cell and thus for toxic activity. Because of this the A chain alone is not significantly toxic.

Please replace the paragraph at page 11, lines 17-30 with the following paragraph:

We originally created a simple tripeptide library inserted in the C-terminal protease-sensitive loop of the SLT-I A chain (Figs. 2A and B). This A chain loop region is naturally constrained due to the presence of a single disulfide bond bridging Cys242 to Cys261 (corresponding to residues 248 to 267 with reference to SEQ ID No.: 1). The maximal diversity of this library can thus be calculated to be 20^3 or 8000 permutations of a tripeptide sequence. As a proof-of-concept that A chain libraries can easily be screened for a new receptor-binding activity, we picked more than 3000 colonies from this A chain-tripeptide library and purified the mutant toxin produced by each clone. We noticed very early in this study that the level of expression of A chain mutant was dramatically increased when expressed in the presence of the wild-type SLT-I B subunit. Thus the mutant forms of A chain were expressed and initially-purified as AB5 toxin variants. Since all A subunits harbor a polyHis purification tag, it is relatively easy to remove the B subunit with denaturants (urea for example) while recovering the A chain on metal-affinity columns or beads. Western blots performed on randomly selected bacterial clones indicated that > 70% of these colonies produced significant amounts of the A chain mutants.

Please replace the paragraph at page 14, line 8 to page 15, line 6 with the following paragraph:

We screened our SLT-I A-heptapeptide library using the cytotoxic function of the A chain as a reporter signal. Cytotoxicity is a more informative property to

measure than binding to a receptor since it implies that the toxin is internalized, processed and delivered near ribosomes, clearly a multi-step event. The cytotoxicity assay was essentially performed as previously described (Bray, et al. (2001) Probing the surface of eukaryotic cells using combinatorial toxin libraries. *Current Biology* 11, 697-701). Briefly, the strategy to screen all our A chain libraries was based on the following principles. Established cancer cell lines such as SK-BR-3 (human breast), CAMA-1 (human breast), 518A2 (human melanoma), PC3 (human prostate) and B16 (murine melanoma) were grown in 96-well plates and used as targets in the primary screen stages. These cell lines were initially selected for our holotoxin library searches (Bray, supra) based on their adherence (plastic), their cell viability staining properties (SRB) in a high-throughput screening setting as well as their lack of receptor and sensitivity to native SLT-1 (to insure a reduced level of false positives). Single bacterial colonies from each library were picked and grown in 96 deep well plates. The cells were harvested, lysed, and their lysates clarified. Since all expressed SLT-1 A chain variants have a 6 histidine tag (SEQ ID No.: 9) at their N-terminus, each of them was purified from their lysate using nickel-affinity beads (96-well format) and layered on target cells. The plates containing the target cells treated with A chain variants were then incubated at 37°C for 48 hours, followed by fixation and staining with Sulforhodamine B (SRB). The SRB assay is a colorimetric endpoint assay, which quantifies viable cells by staining their cellular protein content (Skehan, et al. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 82, 1107-1112.). The SRB assay has been adopted by NCI/NIH for their high-throughput screening of drug candidates on cancer cell lines. Viability assays were repeated for any bacterial extracts leading to cell

death. A first round of screening was performed on more than 5000 bacterial clones (equivalent to 5000 distinct A chain toxins) and 7 toxin variants were identified as repeatable killers of the human melanoma cell line 518A2 (Fig. 5). The abscissa represents the log concentration of toxin used to treat the cells and the ordinate depicts the observed percentage of cells that are viable after 48 hours. The closed triangles depicts the effect of the wild type toxin on 518A2 cells while the two most efficacious A chain variants were termed SAM#3 (open squares) and SAM#5 (X symbols).

Please replace the paragraph at page 15, lines 17-20 with the following paragraph:

The genes coding for the two A chain toxins (SAM3 and SAM5) that resulted in toxicity of the human melanoma cell lines were sequenced to determine the amino acid sequences inserted between residues 245 and 246 of the wild-type A chain (corresponding to residues 251 to 252 with reference to SEQ ID No.: 1). The sequences, including the His-tag, are listed in ~~Seq.~~SEQ ID Nos.: 4 and 5, respectively.

Please replace the Table 1 header on page 16 with the following header:

Table 1: DNA sequences of randomly picked clones from the SLT-1 A chain-tripeptide library (SEQ ID Nos.: 10-20, respectively, in order of appearance). Mutated bases in bold characters. Mutant #41 was identified in our ELISA screen as a strong binder of mAb Onc M27 (Fig. 3).

Please replace the Table 2 header on page 17 with the following header:

Appl. No. 10/598,965
Reply dated November 24, 2009
In response to Office Action dated July 24, 2009

Table 2: Amino acid sequence alignment of randomly selected clones from the SLT-1 A chain- tripeptide library (SEQ ID Nos.: 21-31, respectively, in order of appearance) and ELISA signal of purified SLT-1 A chain variants detected with a mAb (Onc M27) raised against the MUC1 epitope Thr-Arg-Pro. Mutated tripeptide region in bold characters. Mutant #41 was identified in our ELISA screen as a strong binder of mAb Onc M27.

Please replace the Abstract with the following Abstract:

The application relates to libraries of toxin mutants, and to methods of using same in the development of therapeutics targeted against specific cell types.